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RESEARCH PAPER

Is there a temperature-dependent uptake of anandamide into cells?

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Background and Purpose: The temperature dependency of anandamide uptake into cells implies an active mechanism but this is still a matter of considerable debate. We have therefore re-examined the temperature-sensitive uptake of anandamide in ND7/23 mouse neuroblastoma × rat dorsal root ganglion neurone hybrid cells and RBL2H3 rat basophilic leukaemia cells.
Experimental approach: Cellular uptake of [^{3}H] anandamide was measured in the presence of bovine serum albumin at different incubation temperatures and times. Rates of uptake were also measured in wells alone. Free anandamide concentrations were calculated by published methods.
Key results: Anandamide showed a time-dependent saturable uptake into ND7/23 cells. The uptake was greater at 37°C than at 4°C for a given added anandamide concentration following a 5 min incubation. However, this temperature-dependency reflected temperature-dependent effects on the concentration of anandamide available for uptake, rather than the uptake process itself. A similar conclusion could be drawn for the rapid (~1 min) uptake of anandamide into RBL2H3 cells. In contrast, re-analysis of published data for P19 cells indicated a clear temperature-dependency of the uptake at long (15 min) incubation times. The level of anandamide retained by wells alone provided a better measure of free anandamide concentrations than calculated values.
Conclusions and implications: ND7/23 cells may be a useful model system for the study of anandamide uptake. The temperature-dependent uptake of anandamide may reflect effects on free anandamide concentrations rather than on the uptake process itself.
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Keywords: endocannabinoid; anandamide; ND7/23 cells; RBL2H3 cells; cellular uptake; fatty acid amide hydrolase

Abbreviations: AEA, anandamide (arachidonoylethanolamide); AM404, *N*-(4-hydroxyphenyl)arachidonylamide; BSA, bovine serum albumin; FAAH, fatty acid amide hydrolase; KRH buffer, Krebs–Henseleit-bicarbonate buffer; OA, oleic acid; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate

Introduction

The *N*-acylethanolamine compound, anandamide (arachidonoylethanolamide, AEA), is an endogenous signalling lipid that is synthesized upon demand and produces a wide variety of effects in the body as a result of its actions upon cannabinoid and vanilloid (TRPV1) receptors (Devane *et al*., 1992; Zygmunt *et al*., 1999). The action of the compound is short-lived due to effective metabolic pathways, and compounds interfering with the removal of AEA have therapeutic potential in a range of disorders including multiple sclerosis, anxiety, inflammation and inflammatory pain (review see Lambert and Fowler (2005)). The main pathway for AEA breakdown is by cellular uptake followed by hydrolysis to arachidonic acid catalysed by the enzyme fatty acid amide hydrolase (FAAH) (Deutsch and Chin, 1993), although there is evidence that the ability of AEA to act as a substrate for cyclooxygenase-2 and lipoxygenases may be of biological importance (Kozak and Marnett, 2002).

AEA metabolism by FAAH has been well-characterized and selective inhibitors of FAAH are now generally available for experimental study (see Lambert and Fowler (2005)). The process by which AEA is accumulated in cells, on the other hand, has been a matter for considerable debate. Models ranging from energy-independent facilitated transport (Di Marzo *et al*., 1994; Hillard *et al*., 1997), intracellular shuttling and sequestration (Hillard and Jarrahian, 2003; Fowler *et al*., 2004; Ortega-Gutiérrez *et al*., 2004), endocytosis (McFarland *et al*., 2004) to FAAH-driven passive diffusion (Glaser *et al*., 2003) have been suggested. It has also been argued that different cell types may operate different uptake mechanisms, depending upon whether AEA is to be used as a signalling molecule or a source of arachidonic acid (Hillard and Jarrahian, 2005). In this respect, most studies on AEA

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uptake mechanisms have used non-neuronal cells, such as the RBL2H3 basophilic leukaemia cell line. ND7/23 mouse neuroblastoma × rat dorsal root ganglion neurone hybrid cells have a number of 'neuron-like' properties including the expression of sensory neuropeptides and the presence of voltage-activated calcium currents (Wood *et al*., 1990; Kobrinsky *et al*., 1994), and might thus be useful as an easily cultured model of neuronal AEA uptake.

One finding that is consistent with the presence of a designated transport process is the observation that the uptake of AEA is temperature-sensitive (Di Marzo *et al*., 1994; Hillard *et al*., 1997), and the difference between the uptake at 37 and 4°C has been used by several authors (including the present laboratory) as a measure of the 'specific' uptake of AEA. However, the possibility that this temperature sensitivity may reflect differences in the availability of AEA rather than differences in the rate of its uptake, which would make such an estimate of specific uptake invalid, has not been investigated. Issues such as binding to plasticware and formation of micelles have considerable bearing upon the available (as opposed to the added) concentration of AEA in the vicinity of the cells (see Glaser *et al*. (2005)). This highlights the importance of being able to assess the available concentration of AEA in the assay medium. This can, at least in theory, be done when assays are conducted in the presence of fatty acid-free bovine serum albumin (BSA), since the affinity of AEA for BSA is known (Bojesen and Hansen, 2003).

In the present study, we have investigated the ability of ND7/23 cells to accumulate AEA, and determined whether the temperature-sensitive uptake measured in standard AEA uptake assays reflects a sensitivity of uptake or a difference in substrate availability.

Materials and methods

Compounds

Anandamide (arachidonoyl 5,6,8,9,11,12,14,15-^{3}H) (specific activity 7.4 TBq mmol^{-1}), and oleic acid [9,10-^{3}H] ([^{3}H]OA, specific activity 2.22 TBq mmol^{-1}) were obtained from American Radiolabeled Chemicals Inc., St Louis, MO, USA. Unlabelled AEA, was obtained from the Cayman Chemical Co., Ann Arbor, MI, USA. AM404 (*N*-(4-hydroxyphenyl) arachidonylamide) was obtained from Tocris Bioscience, Ellisville, MO, USA. Unlabelled OA, fatty acid-free BSA and normal BSA were obtained from Sigma Aldrich Inc., St Louis, MO, USA.

Culturing of cells

ND7/23 cells (passage range 4–20) were obtained from the European Collection of Cell Cultures, Porton Down, UK. The cells were cultured in DMEM with 2 mM glutamine, 10% foetal bovine serum and 100 U ml^{-1} penicillin + 100 μg ml^{-1} streptomycin. Rat RBL2H3 cells (passage range 13–19) were obtained from the American Type Culture Collection, Manassas, VA, USA). The cells were cultured in MEM with Earl's salts, 2 mM L-glutamine, 15% foetal bovine serum and 100 U ml^{-1} penicillin + 100 μg ml^{-1} streptomycin. Cells were grown in 75 cm^2 culturing flasks at 37°C, 5% CO_2 in humidified atmospheric pressure. Cell culture medium was changed every 2–3 days and passage of cells was performed about twice a week.

Assay of [^{3}H]AEA and [^{3}H]OA uptake

The method of Rakhshan *et al*. (2000) as modified by Sandberg and Fowler (2005) was used. Cells were plated with a density of 2×10^5 cells well^{-1} (unless otherwise stated) in 24-well culture plates and incubated overnight at 37°C with 5% CO_2 in humidified atmospheric pressure. After incubation, cells were washed once with Krebs–Henseleit-bicarbonate (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 0.12 mM KH_2PO_4, 0.12 mM $MgSO_4$ in milliQ deionized water, pH 7.4) containing 150 μM of BSA and once with KRH-buffer alone. Cells were then incubated at the times and temperatures shown with 350 μl of KRH-buffer containing 15 μM (AEA) or 2.25 μM (OA) fatty acid-free BSA (unless otherwise stated) and [^{3}H]AEA or [^{3}H]OA (50 μl, in KRH-buffer containing 15 or 2.25 μM fatty acid-free BSA, respectively) was added to give a final volume of 400 μl well^{-1}. The amount of ethanol present (to dissolve stock solutions of AEA and OA) was in general 3 μl assay^{-1} and was kept constant between samples. It should be pointed out that the process of pipetting samples takes ~20 s per 24-well plate, and this means that the samples to which substrate was first added have a longer incubation time than the subsequent samples. To compensate for this, each experiment was performed in reverse order every other time. After incubation, the plates were placed on ice and washed three times with ice-cold KRH-buffer containing 150 μM BSA (500 μl well^{-1}). The buffer was then removed from the wells and 500 μl of 0.2 M NaOH added. The plates were incubated for 15 min at 75°C after which aliquots (300 μl) of cells were transferred to scintillation tubes and to each tube 4 ml of scintillation fluid was added. Tritium content was assayed by liquid scintillation spectroscopy with quench correction.

Calculation of unbound AEA and OA concentrations

AEA and OA interact with fatty acid-free BSA in such a way that their free (unbound) concentrations can be calculated using the formula $C_{free} = K_d \cdot \nu/(n-\nu)$ where K_d is the dissociation constant for the binding between AEA or OA and BSA at the given temperature, n is a constant (one for AEA, three for OA) and ν, the ratio of the concentration of added AEA or OA to that of the BSA in the medium (Bojesen and Bojesen, 1994; Bojesen and Hansen, 2003). This formula assumes that the free lipid concentration is much lower than the bound lipid concentration. In all the experiments reported here, the ν value is $\leqslant 0.2$, which ensures that the assumption made for calculation of unbound concentrations holds. For AEA, the K_d values at 37, 23, 10 and 0°C are 54.92, 26.05, 11.28 and 6.87 nM, respectively (Bojesen and Hansen, 2003). The K_d value at 4°C was calculated as 8.42 nM from a Van't Hoff replot of these values. Similarly, the K_d values for the binding of OA to BSA calculated from a Van't Hoff replot of values at 0, 10, 17, 23 and 38°C (Bojesen

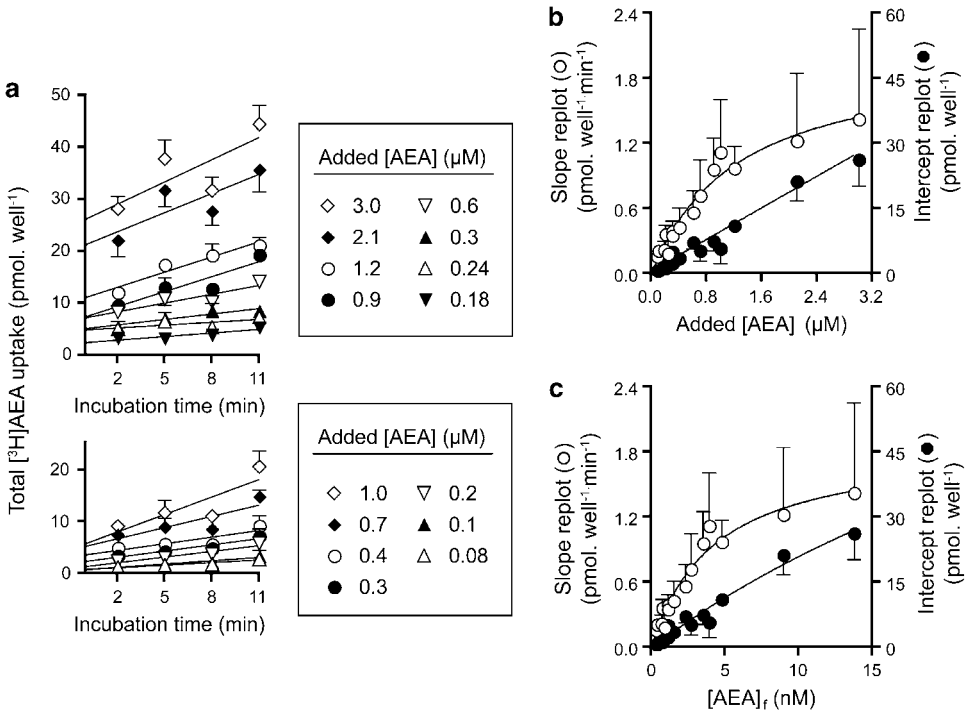

Figure 1 Characteristics of AEA uptake into ND7/23 cells. (**a**) Time courses of the uptake of [^{3}H]AEA. Two series of experiments were undertaken. One using a range of AEA added concentrations of 0.08–1 μM ($n=4$), and one using 0.24–3 μM ($n=4$), both using an incubation temperature of 37°C. Values shown are means$\pm$s.e.m. Linear regression was used to determine slopes and intercepts, which are shown plotted in (**b**) with the added AEA concentrations shown on the abscissa. The error bars in (**b**) are those generated by analyses of the combined data. In (**c**) the data are replotted using the theoretical free AEA concentrations.

and Bojesen, 1994) were 1.4 and 5.6 nM at 4 and 37°C, respectively.

Statistical analyses

Curve fitting, linear regression analyses and statistical comparisons were carried out with the statistical package in the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA). For the kinetic data shown in Figure 1, linear regression of the pooled data set at each substrate concentration was undertaken as described previously (Sandberg and Fowler, 2005). The slopes of the regression lines reflect the time-dependent uptake. The regression lines were also extrapolated back to $t=0$ ('intercept') to give estimates of the initial uptake. These values were then plotted against the AEA concentrations, and are termed 'slope' and 'intercept' replots in this paper. The slope and intercept replots were fitted both to a straight line passing through the origin and to a rectangular hyperbola. The two models were compared by the programme using Akaike's information criterion (AICc) which determines which model is more likely to be correct. The model chosen by this analysis was then used. It should be noted that the method, which is based upon information theory, does not generate a *P*-value, rather relative probabilities that a model is correct. We have included the probability values in the text, so that readers can make their own decisions as to the validity of the model chosen.

Results

Characterization of AEA uptake into ND7/23 cells

Initially, the uptake of AEA into ND7/23 cells was measured at two incubation time points, 2 and 10 min, and the rate of uptake was calculated. This uptake, was, as expected, inhibited by the prototypical uptake inhibitor AM404 (Beltramo *et al*., 1997), whereas the time-dependent uptake of [^{3}H]oleic acid was not (data not shown). Using a series of four incubation times of 2, 5, 8 and 11 min, the saturability of AEA uptake into wells containing ND7/23 cells was investigated at an assay temperature of 37°C (Figure 1a). A large range of AEA concentrations were used, necessitating two series of experiments (shown as separate graphs in Figure 1a). However, there was a good reproducibility for the data with 0.3 μM AEA, which was used in both series. As pointed out in the Materials and methods section, the true incubation times are slightly longer than those shown in the figure, but the difference between times (used to calculate slopes as described below) are exact.

Secondary replots of the data vs added AEA concentration are shown in Figure 1b. The slope replot (i.e. the rate of increase of uptake with time, calculated from the regression lines shown in Figure 1a) was fitted better by a hyperbola than by a straight line passing through the origin. The AICc-derived probabilities of being correct were >99.99 and $<0.01\%$ for the hyperbola and straight line options, respectively, and the K_m value for the slope replot was 1.2 ± 0.2 μM added AEA. The intercept replot (i.e. the extrapolated uptake

values at $t=0$ determined from the same regression lines), on the other hand, was fitted better by a straight line (Figure 1b). In this case, the AICc-derived probabilities of being correct were 18 and 82% for the hyperbola and straight line options, respectively.

In a recent paper (Kaczocha *et al*., 2006), it was reported that the initial uptake of AEA by RBL2H3 cells was non-saturable when plotted against the added AEA concentration, but became saturable when free AEA concentrations, calculated using the formula $K_d \cdot v/(n-v)$, were used. In order to see if the same result was found here, the data shown in Figure 1b was replotted using theoretical free AEA concentrations calculated with this formula. The intercept replot was equally well fitted by a hyperbola as by a straight line passing through the origin (Figure 1c), with AICc-derived probabilities of being correct of 54 and 46% for the hyperbola and straight line options, respectively. The K_m (45 ± 29 nM) value calculated for the hyperbola was rather high (and variable) relative to the AEA concentrations used, but it is consistent with the value of 36 nM found by Kaczocha *et al*. (2006) for RBL2H3 cells. The slope replot showed much more obvious saturability, AICc-derived probabilities of being correct being >99.99 and $<0.01\%$ for the hyperbola and straight line options, respectively, with a K_m value of 4 ± 0.8 nM.

Temperature-dependency of AEA and OA uptake into ND7/23 cells

Parallel to the experiments undertaken at 37°C shown in Figure 1b, wells containing ND7/23 cells were incubated with [^{3}H]AEA for 5 min at 4°C. When the added substrate concentrations were used on the abscissae, the expected temperature sensitivity was seen for AEA (Figure 2a). However, K_d values for the binding of AEA to BSA are highly temperature-dependent (Bojesen and Hansen, 2003) and when the theoretical free AEA concentrations were calculated, the apparent temperature sensitivity was lost (Figure 2b). A loss of temperature-dependency was also seen for OA uptake when theoretical free concentrations were used (Figure 2c and d).

BSA sensitivity of AEA and OA uptake into ND7/23 cells

The data shown in Figure 2 would argue that the apparent temperature dependence of AEA uptake into ND7/23 cells primarily reflects temperature effects upon the free concentration of AEA available for uptake, assuming that the method used to calculate free concentrations is valid for the assay conditions used here. This may not necessarily be the case, since the K_d values were obtained in a very different experimental system (Bojesen and Hansen, 2003).

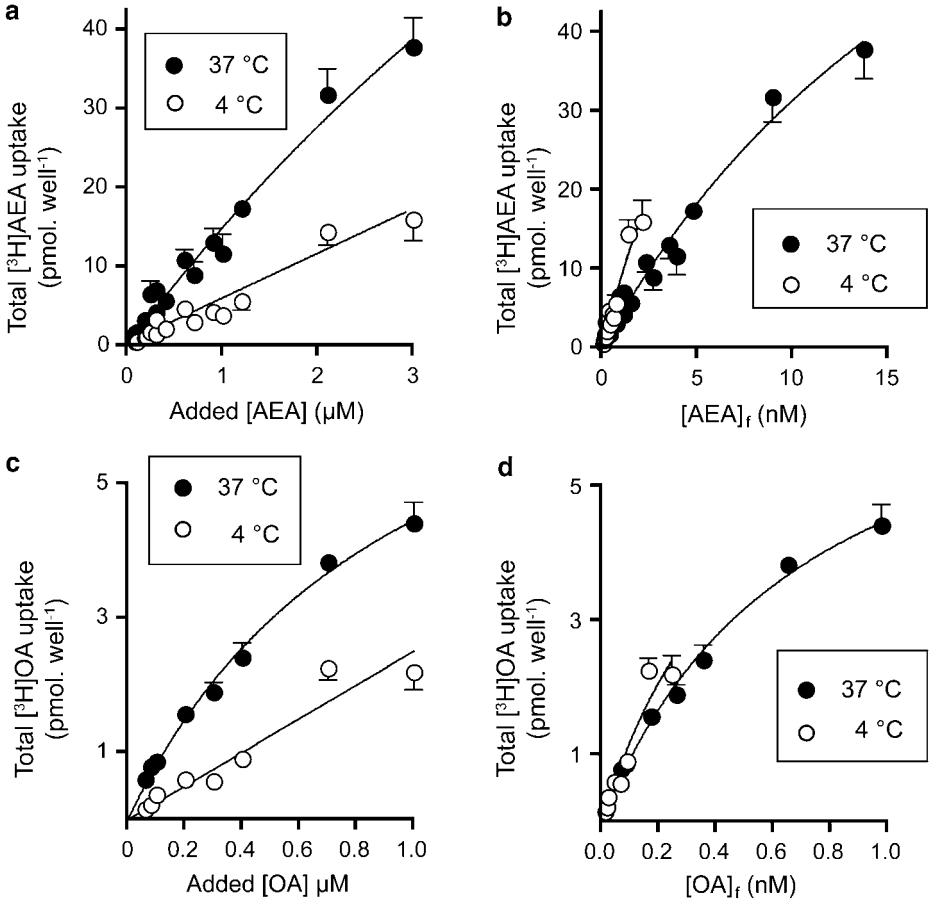

Figure 2 Temperature-sensitivity of the uptake of AEA (**a**, **b**) and OA (**c**, **d**) into ND7/23 cells. An incubation time of 5 min was used, and the data (means ± s.e.m., $n=4$) are replotted either as added AEA/OA (**a**, **c**) or theoretical free AEA/OA (**b**, **d**) concentrations calculated as described in Materials and methods. The AEA data at 37°C shown in (**a**) are the same as the 5 min data points shown in Figure 1a, and can be used here since the data at 4°C were obtained concomitantly.

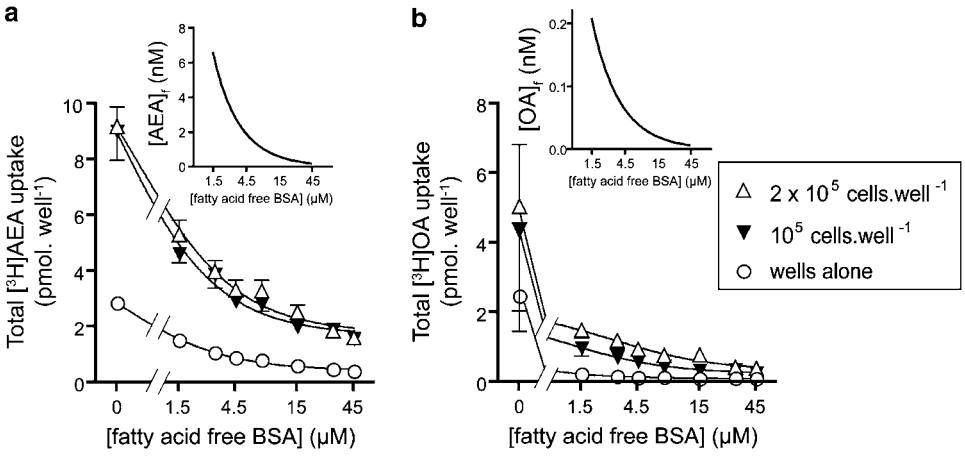

Figure 3 Effect of the assay concentration of fatty acid-free BSA upon the observed rate of uptake of [^{3}H]AEA (**a**) and [^{3}H]OA (**b**) into ND7/23 cells. Values shown are means ± s.e.m. ($n=3$). The added concentration of the substrate was 160 nM and the incubation time was 4 min at 37°C. The inserts show the theoretical free concentrations of AEA and OA, assuming interactions with BSA alone (Bojesen and Bojesen, 1994; Bojesen and Hansen, 2003).

It is thus of importance to investigate the 'performance' of the formula $K_d \cdot \nu/(1-\nu)$ used to calculate free AEA concentrations in different conditions.

In Figure 3a and b, the sensitivity of AEA and OA uptake into ND7/23 cells was investigated using an incubation time of 4 min and added ligand concentrations of 160 nM. Wells alone and wells seeded with ND7/23 cells were found to take up OA in a BSA-sensitive manner (Figure 3b). The retention of OA by the wells alone was very low even at the lowest concentration of BSA tested (where $\nu=0.11$), entirely consistent with the expected unbound OA concentration (shown in the insert to Figure 3b). The cellular uptake of OA was very variable in the absence of BSA, but this variability was lost in its presence, consistent with the notion that the BSA stabilizes the OA in solution.

The uptake of AEA in the presence of cells was greater than for the wells alone at all BSA concentrations tested, although the observed uptake was similar at the two cell densities tested (Figure 3a). It should be pointed out that the cell densities given were those seeded to the wells the day before the assay. With respect to the sensitivity to BSA, AEA was found to behave slightly differently from OA, since a proportion of the uptake remained intact even at high BSA concentrations. When the uptake data shown in Figure 2a in the presence of BSA were analysed using the built-in equation for single site competition of the GraphPad computer programme, a residual uptake (i.e. at [BSA]$\rightarrow\infty$) that was about 15% of that seen in the absence of BSA was found in all three cases.

Temperature sensitivity of rapid AEA uptake into RBL2H3 cells

Given that ND7/23 cells are novel with respect to AEA uptake, it is of course possible that the lack of temperature dependency is a property unique to them. In contrast, the uptake of AEA has been well investigated in RBL2H3 cells (Rakhshan *et al.*, 2000; Jacobsson and Fowler, 2001; Ligresti *et al.*, 2004; Kaczocha *et al.*, 2006) and they were therefore chosen for these experiments. The uptake of AEA by wells alone and by wells containing RBL2H3 cells was investigated at three different assay temperatures (10, 23 and 37°C) and the use of a short (~1 min) incubation time. Unbound AEA concentrations of 0.4, 1 and 3 nM were calculated using the formula $K_d \cdot \nu/(1-\nu)$ and K_d values of 54.92, 26.05 and 11.28 nM for 37, 23 and 10°C, respectively (Bojesen and Hansen, 2003). The data is shown in Figure 4a. At each tested theoretical free AEA concentration, and for both wells alone and wells seeded with 2×10^5 RBL2H3 cells, the uptake was in rank order 10 > 23 > 37°C.

Given that the binding of AEA to plastic wells is presumably a low-affinity, high-capacity process, it would be expected that the binding of [^{3}H]AEA to the wells alone should be directly proportional to the available AEA concentration. Our laboratory has previously reported the binding of AEA to wells at a temperature of 37°C and an incubation time of 45 s using the same assay as in the present study (Sandberg and Fowler, 2005). Re-examination of that data indicated that over a concentration range of 0.1–1 μM added AEA, a very good linear relationship was seen between added AEA and AEA bound to wells; analysis of the mean data shown in Figure 4a of Sandberg and Fowler (2005) gave a r^2 value of 0.989, and the line passed through the origin. We could confirm this result in the present study using a range up to 3 μM added AEA, and demonstrated further that the observed 'uptake' to the wells was temperature dependent (Figure 4b), a result in line with a study using a single concentration (100 nM) of AEA and an incubation time of 4 min in the absence of BSA (Ortega-Gutiérrez *et al.*, 2004). A plot of the observed uptake for cells against the binding to the wells alone should thus be a good measure of the uptake at different available AEA concentrations. When this was done for the data from the RBL2H3 cells, the values were well fitted to a straight line passing through the origin and there was no evidence of a temperature dependency (Figure 4c).

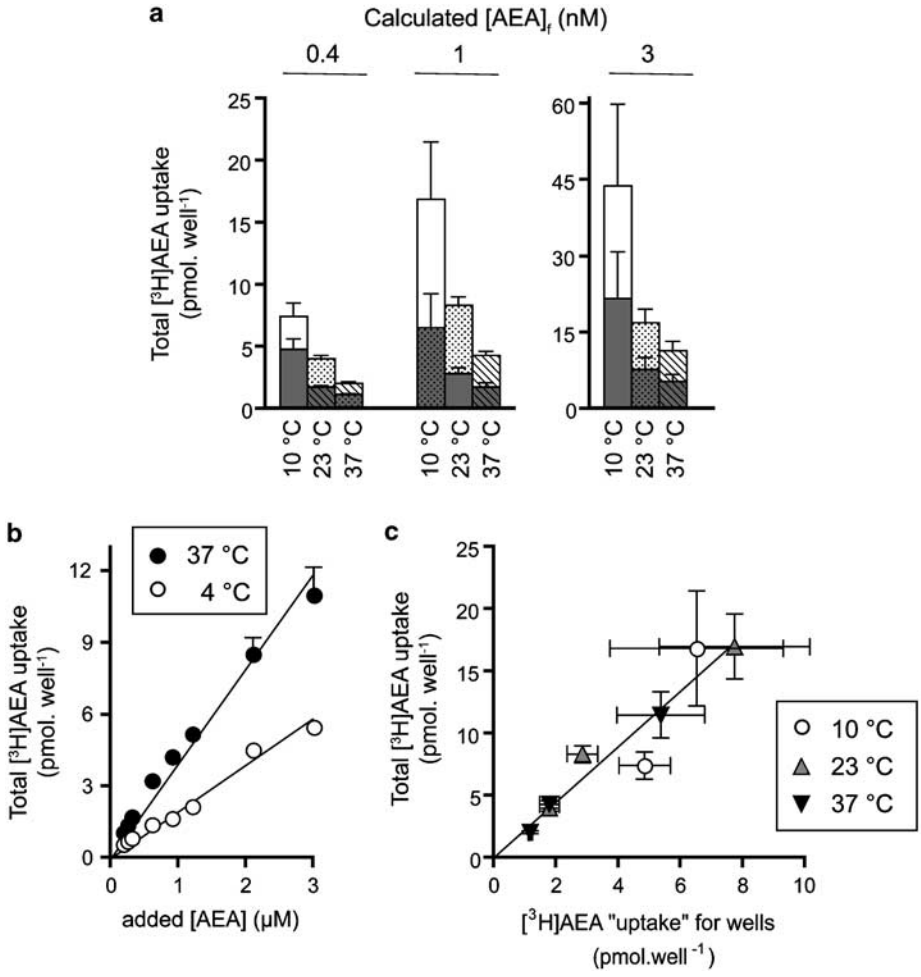

Figure 4 (**a**) Temperature dependent uptake of [^{3}H]AEA into RBL2H3 cells. An incubation time of $\sim$1 min was used. The added concentrations of AEA were calculated assuming an interaction with BSA alone with the binding characteristics and K_d values described by Bojesen and Hansen (2003). Thus, for example, a free concentration of 0.3 nM required added concentrations of 0.51, 0.23 and 0.11 μM at incubation temperatures of 10, 23 and 37°C, respectively. Complete columns represent uptake into wells seeded with 2×10^5 RBL2H3 cells; the darker columns enclosed within the columns represent uptake into the wells alone. Values shown are means $\pm$ s.e.m. ($n = 4$). (**b**) 'Uptake' of [^{3}H]AEA by wells alone at 37°C and 4°C. An incubation time of 5 min was used. Data are means of a single experiment; the error bars are for the six replicates. (**c**) Correlation between the observed uptake for wells seeded with RBL2H3 cells and for wells alone. The r^2 value for the regression line, which was determined from the mean values of all the data points shown in the figures, was 0.91. The highest added AEA concentration tested (3.51 μM at 10°C) was excluded from the figure since it is considerably to the right of the other data points and will thus exert an unacceptable bias in the analysis. Indeed, if the point was included, the r^2 value of the regression line (which had a similar slope to that shown in the figure) increased to 0.98 (data not shown).

The ratio of the slopes for the regression lines shown in Figure 4b is 0.49. This value can be used to provide an assessment of the relative concentrations of AEA at 4 and 37°C for the temperature sensitivity experiments with ND7/23 cells shown in Figure 2a. When this is done, the two curves become essentially superimposable (Figure 5a), suggesting that regardless of the measure used to assess the AEA concentration available for uptake, the ND7/23 cells do not show a temperature-sensitivity at this incubation time. This laboratory has previously published time-dependency data for P19 cells using the same assay conditions as here (Sandberg and Fowler, 2005). When the data from Figure 4b and c of that paper were replotted compensating for the temperature-dependent effect on AEA concentrations, the temperature-dependency was small at 4 min incubations, but was marked at 15 min incubations (Figure 5b and c).

Discussion

The mechanism(s) responsible for the cellular uptake of AEA are a matter of considerable current controversy. The present study has primarily focussed upon methodological issues, and there are three main findings:

Firstly, ND7/23 cells may be a useful model for the 'neuronal' uptake of AEA. One aim of the present study was to establish whether or not ND7/23 cells can be used to study the uptake of AEA in a cell line that is straightforward to culture and has characteristics of sensory neurons. The cells behave very much as would be hoped, in as much as they show a time-dependent uptake of AEA that is saturable and that can be inhibited by the standard AEA uptake inhibitor AM404. There is some specificity in this process, since AM404 does not affect OA uptake. One observation

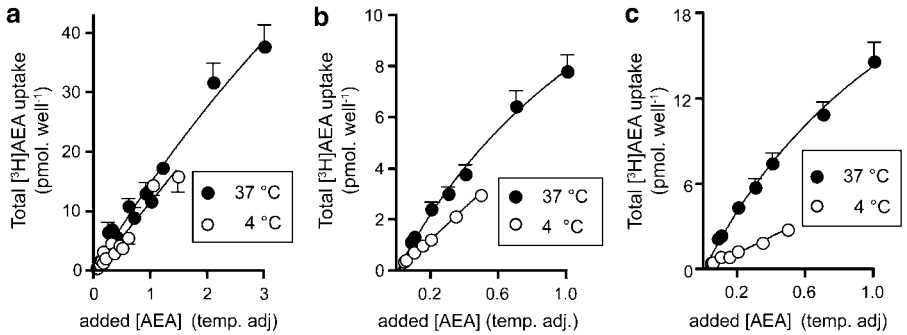

Figure 5 Re-examination of the effect of assay temperature upon the uptake of AEA into ND7/23 cells (**a** incubation time 5 min), P19 cells (**b**, **c**, incubation times 4 and 15 min, respectively). The concentrations of AEA at 37°C are those added, while the concentrations of AEA at 4°C are those added $\times$ 0.49 (the ratio of the slopes for the regression lines shown in Figure 4b). The data for the ND7/23 cells is taken from Figure 2a, while the data for P19 cells are taken from Figure 4b and c of Sandberg and Fowler (2005).

of note is that whilst the observed uptake in the presence of cells was greater than for wells alone, the rate of uptake was similar for wells seeded with two different densities. It should be pointed out that the cell densities given were those seeded to the wells the day before the assay. More importantly, simple subtraction of the values for cells minus the values for the wells alone to give an estimate of the cell-dependent uptake is not an option in these conditions, since the cells will cover, and hence reduce the well 'uptake'. This was shown elegantly in the study of Ortega-Gutiérrez *et al.* (2004) where in the absence of BSA, the 'uptake' for wells alone was in fact considerably higher than the uptake for wells containing cultured neurons. These two factors presumably explain why the two cell densities show similar rates of uptake.

Secondly, measurement of the retention of AEA by wells alone may provide a better measure of the available AEA concentration in the presence of BSA than the use of the formula $C_{free} = K_d \cdot v/(1-v)$, to calculate unbound AEA concentrations. This formula assumes that all the added AEA can be bound to BSA. The residual uptake in the presence of high BSA concentrations would suggest that this is not the case, in contrast to the situation for OA. The binding of AEA to the wells, on the other hand, is presumably a high-capacity, low-affinity process, and thus will be proportional to the available concentration of AEA in the medium. Our data are entirely consistent with this view. Given that binding to plastic and hence available [AEA] is directly proportional to the added AEA (at least for v values $\leqslant 0.2$, which was the range we investigated) our study would argue that there is as yet no convincing evidence for saturability of the initial phase of AEA uptake into either ND7/23 cells (present study), P19 cells (Sandberg and Fowler, 2005) or RBL2H3 cells (Ligresti *et al.*, 2004; Kaczocha *et al.*, 2006), in contrast to the clear saturability seen in these cell lines at longer incubation times. This does not, however, mean that initial saturable processes do not occur (such as would be inferred from experiments studying AEA transport by erythrocyte ghosts, where the presence of an unstirred layer in the vicinity of the membrane may provide a diffusion barrier (Bojesen and Hansen, 2006)), merely that they are not measurable under the conditions used.

The retention of AEA by the wells has been used to advantage here as a measure of AEA concentrations available for uptake, but it is generally a nuisance, particularly when it is considered that uptake inhibitors like AM404 and UCM707 prevent this retention at concentrations similar to those affecting uptake (Karlsson *et al.*, 2004; Ortega-Gutiérrez *et al.*, 2004). It is possible that the component of the wells can be minimized not only by the use of BSA, but also with different culture plates, or by different ways of collecting the cells. To our knowledge, this has not been investigated systematically, but would certainly be a worthwhile exercise.

Thirdly, temperature-dependent uptake of AEA may under some conditions reflect the use of added, rather than available AEA concentrations. Many authors using different cells and different incubation times have reported temperature-dependent uptake of AEA (see e.g. Di Marzo *et al.* (1994), Jacobsson and Fowler (2001), Ruiz-Llorente *et al.* (2004), Oddi *et al.* (2005)). The data presented here would suggest that for RBL2H3 cells (incubation time 1 min) and ND7/23 cells (incubation time 5 min), the observed temperature dependency is primarily due to temperature-dependent variations in the concentration of AEA available for uptake rather than the uptake process itself. Consistent with this result, inspection of the data in Figure 2c and e of the study of Kaczocha *et al.* (2006) indicates that the slope of the uptake of AEA into RBL2H3 cells at 37°C at an incubation time of $\leqslant$25 s is approximately double that seen at 0°C, which is again entirely consistent with our data for ND7/23 cells and with the temperature-dependency of the binding to plastic.

Temperature-dependent effects unrelated to substrate availability, however, can be demonstrated at long incubation times. Thus, for the P19 cells, a small temperature-dependent component of the uptake is observable at a 4 min incubation time, and this is greatly increased when the incubation time is lengthened to 15 min, suggesting that this reflects an intracellular event. In many (but not all, see e.g. Beltramo *et al.*, 1997; Ruiz-Llorente *et al.*, 2004) cells, it has been found that FAAH drives the AEA uptake (Deutsch *et al.*, 2001; Day *et al.*, 2001; Glaser *et al.*, 2003; Kaczocha *et al.*, 2006). Such an enzymatic component should be sensitive to a drop in assay temperature, so it would be expected that for a cell line involving FAAH in AEA uptake (such as P19 cells, Sandberg and Fowler, 2005) a temperature sensitivity will become apparent as the incubation time is increased.

Whether or not FAAH alone provides this temperature sensitivity requires investigation.

From the above discussion, it can be concluded that at short incubation times, temperature-dependency of the uptake process itself cannot unequivocally be demonstrated with 'standard' uptake assays. It is of course possible that a temperature-dependent uptake can be distinguished at very short incubation times using other assay techniques. Indeed, the very rapid efflux of AEA from erythrocyte ghosts is a case in point. At an assay temperature of 0°C and a $[BSA]_i$ of 15 μM, the rate constant for the transfer of AEA from an AEA–BSA complex within the ghost to the outer surface of the ghost membrane is $0.117 \pm 0.018\,s^{-1}$ (Bojesen and Hansen, 2005). At higher assay temperatures, the rate of efflux was so fast that the authors could not measure it reliability with the techniques at their disposal (Bojesen and Hansen, 2005). A half-life for translocation through the membrane of $\sim$16 s at 4°C estimated by these authors would mean that at incubation times $\geqslant$1 min (assuming the same processes occur in the other direction, and are seen in other cells), this particular process would be >92% completed at 4°C, and so a temperature-dependency would not be seen. Thus, their data is not inconsistent with the present study.

A final word is perhaps appropriate concerning the nature of the uptake process under study. As pointed out above, there seems to be a consensus emerging that, in contrast to the time-dependent uptake, the initial rate of uptake shows very little saturability. Studies in several cell lines have suggested that the initial uptake is, in addition, not sensitive to uptake inhibitors like AM404 (Glaser *et al*., 2003; Sandberg and Fowler, 2005). Given that AEA can cross biological membranes very rapidly indeed (Bojesen and Hansen, 2005), it is not unreasonable to conclude that the time-dependent AEA uptake measured in standard uptake assays of the type used here probably are investigating the intracellular redistribution of AEA rather than a transport across the plasma membrane. The last word on this matter, however, has certainly not been said.

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Conflict of interest

The authors state no conflict of interest.

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